THE ACTION OF HEAT AND MOISTURE ON LEATHER[1]

PART VI. DEGRADATION OF THE COLLAGEN

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SUMMARY

Collagen and tanned collagens have been adjusted to various pH values and stored at 40°C. at different relative humidities. Hydrolytic breakdown of the protein as indicated by release of N-terminal residues increases with decrease in pH and with increase in relative humidity.

When breakdown becomes extensive, denaturation follows and this in its turn accelerates further hydrolytic action.

Cross-linking of the protein by a tanning agent retards hydrolytic breakdown during storage and also helps to maintain structure even when such breakdown is quite extensive.

Oxidative breakdown is also thought to occur but no direct evidence of the presence of keto acids, one of the expected by-products of such degradation, was obtained.

The implications of these findings to the deterioration of leather on exposure to warm moist conditions or acid atmospheres, and to the storage of pickled pelts are discussed.

INTRODUCTION

It has been shown in earlier papers in this series that degradation of the protein in many cases plays an important part in the deterioration of leather by moist heat (1–4). Such degradation may take either of two forms, (1) denaturation, i.e. loss of the three chain helical structure such as occurs on the conversion of collagen to gelatin or (2) breakdown of the individual polypeptide chains. Both probably occur to some extent but their relative importance and detailed information on how they are affected by pH, humidity, etc. are lacking.

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[2]Now in charge of the Film Archive Laboratory at the Imperial War Museum.

The most likely cause of scission of the polypeptide chains is hydrolytic breakdown with release of α-amino and carboxyl groups. Preliminary investigations using the fluorodinitrobenzene technique (4) for the detection of α-amino groups indicated that such breakdown occurred to some extent even at pH values normally considered safe for leather. The present paper reports the extension of this work to collagen and tanned collagen stored under a variety of conditions of pH and humidity using an improved and more rapid procedure for the determination of N-terminal residues.

The possibility of breakdown involving oxidative deamination was also indicated in the earlier investigation (4). This might occur by a mechanism similar to that postulated for the breakdown of proteins on exposure to U.V. or γ-radiation (5–7) i.e.

$$-\mathrm{CH}(R_1)\cdot\mathrm{CO}\cdot\mathrm{NH}\cdot\mathrm{CH}(R_2)\cdot\mathrm{CO} \rightarrow -\mathrm{CH}(R_1)\cdot\mathrm{CO}\cdot\mathrm{NH_2} + \mathrm{O{:}C}(R_2)\cdot\mathrm{CO}-$$

Attempts were therefore made to detect and determine the keto acids which are a likely by-product of such breakdown. Unfortunately the results were inconclusive since the high blanks obtained with the original collagen tended to obscure any increases due to breakdown.

EXPERIMENTAL

Materials

Collagen.—The collagen preparation described in Part V (4) was used throughout. This was ground to pass 20 mesh.

Chrome Tanned Collagen.—Samples of collagen were tanned at two levels of chromic oxide. 100 g samples of the collagen powder were soaked back in 800 ml five percent Na_2SO_4 and sulfuric acid added to bring the pH to 3. 120 or 12 ml of an SO_2-reduced chrome liquor containing 8 g Cr_2O_3 per 100 ml were added, the suspension was shaken for eight hours and left overnight. The next day the pH was raised slowly to pH 4.0 by the addition of two percent $NaHCO_3$ and the suspension again left overnight. The chrome tanned powder was washed thoroughly with tap water and then with two changes of distilled water. It was acetone dehydrated for storage.

The final chromic oxide contents of the two samples were 4.0 and 0.57 percent.

Glutaraldehyde Tanned Collagen.—Two levels of glutaraldehyde were offered, ten and 2.5 percent on dry weight. Forty grams collagen powder were soaked back in 480 ml water and 50 g Na_2SO_4 added, followed by 16 or four ml 25 percent glutaraldehyde solution. After stirring for 30 minutes, 4.2 g Na_2HPO_4 and 9.6 g KH_2PO_4 were added to bring the pH to 6.5. The suspension was stirred for a further two hours and then left overnight. The powder was filtered off,

washed by decantation and finally acetone dehydrated. The uptake of glutaraldehyde, calculated from analysis of the liquors before and after tanning, was 9.0 and 2.0 percent respectively.

Treatment of Samples for Storage

Buffered Collagen and Glutaraldehyde Tanned Collagen.—Ten gram samples were shaken with 200 ml. 1.0 M citrate buffers of pH 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0 for 24 hours. 0.1 percent Nipasept (ester compounds of p-hydroxy benzoic acid) was added to discourage microbiological attack. Similar series in which ten percent NaCl was added to reduce swelling were also prepared. The concentrations of acid used and the pH of water extract are summarized in Table VI.

Storage of Samples

The samples of collagen buffered with citrate were spread thinly on aluminum trays and stored in closed glass vessels at 40°C. for eight weeks. The following conditions were used to maintain the different relative humidities.

1. Over water — 100 percent r.h.
2. Over sat'd Na_2SO_4 solution — 85 percent r.h.
3. Over sat'd NaCl solution — 75 percent r.h.
4. Over sat'd K_2CO_3 solution — 40 percent r.h.
5. Dry — open jars

The other samples were stored in glass trays over water only. A little toluene was added to discourage microbiological attack. After storage the samples were air dried at room temperature.

Analytical Methods

pH of Water Extract.—A 2.5 g sample was placed in 50 ml distilled water for 24 hours (9). The pH was determined on the extract.

Chromium.—Digestion with perchloric acid followed by titration with ferrous ammonium sulfate (10).

Glutaraldehyde in Solution.—Formation of the bisulphite complex followed by determination of the iodine required to oxidize the complex by back titration with thiosulfate (11).

Determination of N-Terminal Residues

Reaction with 1-fluoro-2, 4-dinitrobenzene (FDNB).—Between 0.2 and 1.0 g of the air dry collagen powders were suspended in 20 ml saturated sodium bicarbonate solution and 0.2 ml FDNB added. The reaction mixture was kept in the dark at room temperature for three days with occasional shaking. The solid was then filtered off, washed with ether to remove excess FDNB and hydrolyzed with 6 *N* HCl in a sealed tube at 105°C. for 16 hours. The filtrate was

also extracted with ether, neutralized by the addition of a few drops conc. HCl, evaporated down to about five ml and hydrolyzed with an equal volume of conc. HCl as above.

Separation and Determination of α-DNP-Amino Acids.—The general procedure was based on the method described by Steven and Tristram (12). The α-DNP-amino acids were extracted from the hydrolyzates with ether, and dinitrophenol, dinitroaniline and other breakdown products were removed on a column of silica gel. The α-DNP-amino acids were then separated by two dimensional paper chromatography on Whatman No. 1 paper using n-butanol saturated with 0.1 percent NH_4OH (13) followed by 1.5 *M* phosphate buffer (156 g $NaH_2PO_4 \cdot 2H_2O$ + 89 g $Na_2HPO_4 \cdot 2H_2O$ per liter) (14).

The spots were cut out and extracted with one percent $NaHCO_3$ in 25 percent v/v aqueous ethanol in stoppered tubes for 18 hours at 40°C. The extracts were centrifuged to remove fragments of paper and the optical density at 360 mμ measured. Separation of DNP-phenylalanine from dinitrophenol presented some problems in the later stages of the investigation when a new batch of chromatography paper was put into use. However, this did not materially affect the values for total N-terminal residues released.

The amounts of the α-DNP-amino acids were calculated by reference to standard curves obtained using authentic DNP-amino acid derivatives (see Part V [4]). Exposure to light was kept to a minimum throughout the manipulations to reduce losses (see Chromatographic and Electrophoretic Techniques [15]).

The amount of collagen in each portion of the reaction mixture (solid and filtrate) was calculated from micro-kjeldahl nitrogen (4) determinations carried out on suitable aliquots of the hydrolyzates taken after the extraction of the DNP-amino acids with ether. The dinitrophenyl group on the lysine remaining in the aqueous fraction did not contribute to the total nitrogen as determined by the standard micro-kjeldahl method.

The nitrogen content of the collagen was taken as 17.8 percent (4) and N-terminal residues were calculated as millimoles per 100 g collagen using the appropriate correction factors for losses during hydrolysis and separation (see below).

Replicate determinations agreed within ten percent, except for glycine. The wider variations with this amino acid are presumably associated with the large and rather variable losses occurring during hydrolysis.

Recovery of DNP-Amino Acids.—Losses of DNP-amino acids in the actual chromatographic separation and determination procedures varied between zero and five percent. Losses during hydrolysis were much larger and were dependent on the conditions of hydrolysis and time and method of separation.

Preliminary tests indicated that losses were less with 6 *N* than with 12 *N* HCl and all hydrolyses were, therefore, carried out using the former.

TABLE I

RECOVERY OF DNP-AMINO ACIDS

Material Hydrolyzed	1 g Collagen + ca. 70 μg of DNP-Amino Acid	1 g Collagen + 0.025 g DNA* + 0.025 g DNPOH† + ca. 70 μg of DNP-Amino Acid	1 g DNP Collagen + ca. 70 μg of DNP-Amino Acid	1 g DNP Collagen + ca. 140 μg of DNP-Amino Acid	1 g DNP Collagen + ca. 280 μg of DNP-Amino Acid	Values Used for Actual Determinations
DNP-Amino Acid Added			Percent Recovered			
Aspartic			47			47
Glutamic	39	58	66	59	66	66
Serine	28	54	64	53	60	63
Threonine	35	68	63	—	—	63
Glycine	9	16	47	63	54	47
Alanine	30	—	86	—	—	86
Valine	57	78	81	87	88	88
Leucine	33	72	75	—	—	75
Phenylalanine	32	71	63	—	—	64
bis-lysine	19	61	62	—	—	61

*DNA = dinitroaniline.
†DNPOH = dinitrophenol.

The influence of breakdown products of the DNP-protein, e.g. dinitrophenol and dinitroaniline, on the recovery of DNP-amino acids was examined. Recovery was much improved by addition of dinitroaniline and dinitrophenol, and, in most cases, further improved in the presence of the DNP-protein. The recoveries obtained in the presence of DNP-collagen (1 g DNP protein + 70 μg DNP-amino acid — column 4) were used in calculating results, since these amounts approximated most nearly to the proportions found in practice.

Determination of Carbonyl Compounds

The method used followed the general procedure described by Lappin and Clarke (16) using 2,4-dinitrophenyl hydrazine (DNPH), and pyruvic acid as the basic standard.

Reaction of collagen directly with DNPH followed by acid hydrolysis led to the production of numerous artifacts and attempts to separate the phenyl hydrazines from the hydrolyzate by solvents as suggested by Kawano, Katsuki, Yoshida and Tanaka (17), only produced intractable emulsions.

Reaction after hydrolysis gave more promising results but unfortunately the original collagen gives a high blank due to decomposition of serine and threonine. In attempts to reduce this blank and improve the method the acid concentrations were varied between 2 and 12 *N* and the times between four and 40 hours. The blank increased with the concentration of acid and time of hydrolysis and results were not very reproducible.

TABLE II

EFFECT OF HYDROLYSIS CONDITIONS ON DETERMINATION OF CARBONYL GROUPS USING DINITROPHENYLHYDRAZINE

(Millimoles per 100 g Air Dry Collagen as Pyruvic Acid)

Normality of Acid	2 *N*	6 *N*	12 *N*
Hydrolyzed 4 hours	0.87	0.81	0.82
Hydrolyzed 16 hours	1.53	1.59	1.95
Hydrolyzed 40 hours	2.05	2.07	3.46

The procedure finally adopted was hydrolysis in 2 *N* HCl for 16 hours at 105°C. followed by dilution to 1 *N* acid concentrations before reaction with dinitrophenylhydrazine.

EXPERIMENTAL RESULTS

Collagen Buffered with Citrate

Appearance and Solubility.—In the first series of experiments collagen samples were buffered with 1 *M* citrate to pH values in the range 2.5–5.0 and stored for

eight weeks at 40°C. either dry or at relative humidities of 40, 75, 85 and ca 100 percent.

The pH values quoted throughout are for the pH of water extract of the leather (2.5 g in 50 ml).

The samples stored at pH 2.5 or 3 at the two highest humidities were badly damaged during storage, losing all their fibrous structure and becoming gelatinous in appearance. Two other samples, that buffered to pH 3.5 and stored at 100 percent R.H. and that buffered to pH 2.5 and stored at 75 percent R.H. were beginning to show similar damage but the rest, although slightly discolored, retained their fibrous appearance. In spite of the extensive breakdown in some cases, changes in pH were small, generally less than 0.1 pH unit.

The four most damaged samples dissolved almost completely in the bicarbonate buffer in which the reaction with FDNB was carried out and only a small amount was reprecipitated during the reaction. The sample stored at 100 percent R.H. and pH 3.5, also that stored at 75 percent R.H. and pH 2.5 dissolved to an appreciable extent (see Table III). Solubility in citrate buffer was also determined in a few cases but as it was found to follow the same general pattern as solubility in the bicarbonate buffer it was discontinued.

Hydrolytic Breakdown — N-Terminal Residues.—The results of determinations of N-terminal residues are given in Table III and summarized graphically in Figure 1. At pH 4.0 and above, relatively few N-terminal residues were liberated even at the highest humidity. As the pH of the collagen was decreased, however, the number liberated increased sharply in the samples stored at 100 percent R.H. until at pH 3.0 it reached 9.4 millimoles per 100 g, compared with only 0.7 at pH 4.0 and 1.8 at pH 3.5. At 85 percent R.H., breakdown followed the same general trend but was appreciably less; at 75 percent R.H. the sharp increase was not apparent until the pH fell below 3.0 and at pH 2.5 the number of N-terminal residues released was only about one quarter of that released at 100 percent R.H. At 40 percent R.H. and "dry" very few N-terminal residues were liberated even at pH 2.5, but the DNP derivative was soluble to the extent of 14 percent indicating that some other type of breakdown may have occurred.

The same range of N-terminal residues, in roughly the same proportions, was found in all cases, whether the breakdown was large as at pH 2.5 or smaller as at the higher pH values. Detailed results for four of the collagen samples are given in Table IV. Glycine predominates as an N-terminal residue in each case followed by alanine and then generally by aspartic acid, serine and phenylalanine. If the results are considered in relation to the total number of residues of the amino acid present in collagen, phenylalanine is always found in much larger amounts than would be expected from random breakdown. (Separation of DNP-phenylalanine from dinitrophenol was satisfactory at this stage, see above). For example, in the collagen stored over water at pH 2.5, one in twelve of the peptide bonds involving the amino group of phenylalanine is broken compared with only one in 75 of those involving glycine. Glycine and alanine are, in fact, among

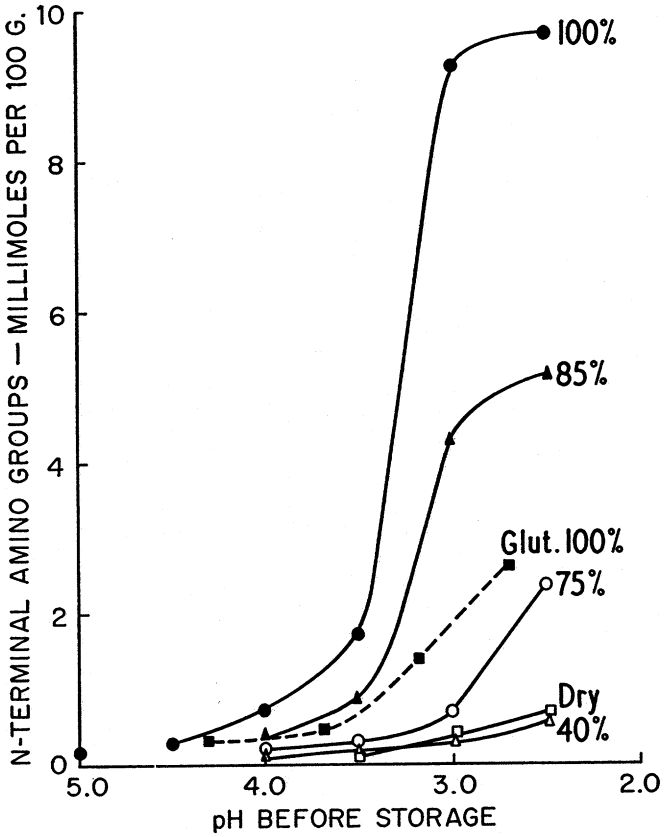

FIGURE 1.—N-terminal residues in collagen and glutaraldehyde tanned collagen stored at 40°C. for 8 weeks — buffered with citrate.

Collagen stored at 100% r. h.
Collagen stored at 85% r. h.
Collagen stored at 75% r. h.
Collagen stored at 40% r. h.
Collagen stored Dry
Glutaraldehyde tanned Collagen stored at 100% r. h.

those contributing fewest N-terminal residues in relation to the total amounts present; serine, threonine and aspartic acid on the other hand follow phenylalanine in contributing more than the average proportion.

The mean weight per mole of N-terminal residue (M_{TR}) i.e. the mean average chain weight, has also been calculated for the soluble and insoluble fractions. This gives an indication of the molecular size. The M_{TR} of the insoluble fraction decreases from about 600,000* for the original material to about 9,000 in the

*Even though the material has been limed this figure is six times greater than that corresponding to the three chain helical model of molecular weight about 300,000 tropocollagen now generally accepted for the collagen macromolecule. The recent report by Hörmann, Joseph and von Wilm (18) on the presence of N-acetylated residues offers a possible explanation.

TABLE III

SOLUBILITY AND MEAN WEIGHT PER MOLE OF N-TERMINAL

(Residues for Buffered Systems Stored at 40°C. and 100% R. H.)

Initial pH	2.5	3.0	3.5	4.0	4.5	5.0
Collagen						
Solubility — percent						
Dry	14	3	0.4	—	—	—
40% r. h.	12	0.6	0.3	0.1	—	—
75% r. h.	59	4	0.7	2.0	—	—
85% r. h.	100	76	6	4	—	—
100% r. h.	76	80	66	24	5	2
Mean Weight per Mole of N-Terminal Residue — M_{TR}						
Dry						
Soluble fraction	92,000	48,500	—	—	—	—
Insoluble fraction	191,000	291,000	595,000	—	—	—
40% r. h.						
Soluble fraction	110,000	13,100	5,500	2,600	—	—
Insoluble fraction	206,000	375,100	552,100	662,000	—	—
75% r. h.						
Soluble fraction	34,200	14,100	9,000	46,600	—	—
Insoluble fraction	63,000	214,000	394,000	410,000	—	—
85% r. h.						
Soluble fraction	19,400	20,900	13,800	22,300	—	—
Insoluble fraction	nil	36,000	196,000	435,000	—	—
100% r. h.						
Soluble fraction	11,000	9,700	69,000	97,000	63,000	48,000
Insoluble fraction	8,500	16,800	42,500	156,000	374,000	420,000
Glutaraldehyde — high level						
Initial pH	2.7	3.2	3.7	4.3	—	—
Solubility, %	10	4	1	0.8	—	—
M_{TR}						
Soluble fraction	11,700	8,200	nil	nil	—	—
Insoluble fraction	51,300	109,000	250,000	450,000	—	—

TABLE IV

TYPICAL VALUES FOR N-TERMINAL RESIDUES OF DETERIORATED COLLAGEN

(Expressed in all Cases as m. moles per 100 g Original Total Collagen)

	pH 2.5			pH 4.0		
	DNP-Fraction			DNP-Fraction		
	Insoluble	Soluble	Total	Insoluble	Soluble	Total
Stored at 100% Relative Humidity, 8 weeks at 40°C.						
Solubility, %*	24	76		76	24	
Aspartic acid	0.22	0.43	0.65	0.040	0.046	0.086
Glutamic acid	0.10	0.24	0.34	0.015	0.010	0.025
Serine	0.21	0.61	0.82	0.048	0.018	0.066
Threonine	0.12	0.26	0.38	0.018	0.006	0.024
Glycine	1.10	3.14	4.24	0.20	0.11	0.31
Alanine	0.37	1.07	1.44	0.065	0.029	0.094
Valine	0.09	0.21	0.29	0.016	0.006	0.022
Leucines	0.17	0.22	0.39	0.024	0.010	0.034
Phenylalanine	0.34	0.69	1.03	0.052	0.014	0.066
bis-lysine	0.11	0.05	0.16	0.015	trace	0.015
Total			9.74			0.742
Mean wt. per mole of N-terminal residue	8,500	11,000	10,300	156,000	97,000	135,000
Stored at 40% Relative Humidity, 8 weeks at 40°C.						
Solubility, %*	87	12		99.9	0.1	
Aspartic acid	0.027	0.019	0.046	0.021	0.003	0.024
Glutamic acid	0.019	0.018	0.037	0.010	0.002	0.012
Serine	0.062	0.016	0.078	0.032	0.011	0.043
Threonine	0.036	0.014	0.050	0.013	0.009	0.022
Glycine	0.12	trace	0.12	0.036	trace	0.036
Alanine	0.040	0.021	0.061	0.009	0.008	0.017
Valine	0.018	trace	0.018	0.002	trace	0.002
Leucines	0.023	trace	0.023	0.008	trace	0.008
Phenylalanine	0.057	0.028	0.085	0.011	0.008	0.019
bis-lysine	0.025	trace	0.025	0.009	trace	0.009
Total			0.541			0.192
Mean wt. per mole of N-terminal residue	206,000	110,000	185,000	662,000	2,600	520,000

*Solubility of DNP-derivatives in sodium bicarbonate solution during reaction with FDNB. (Nitrogen dissolved as percent total nitrogen.)

damaged samples, while that of the soluble fraction first increases and then decreases. Presumably the small amounts of material dissolved from the relatively undegraded samples represents small polypeptides present in the collagen as a result of liming. As the pH is decreased and the humidity rises, larger peptides are broken off and appear in the soluble fraction and, finally, under still more drastic conditions these are themselves hydrolyzed further. Under such conditions the M_{TB} of the soluble and insoluble fractions approach one another indicating a general overall breakdown.

At the high humidities and low pH values it was obvious that some of the collagen samples were denatured during storage and that the increases in solubility were at least partially due to this. The first signs of denaturation were generally associated with a marked increase in N-terminal residues. The question of the part played by denaturation in release and detection of N-terminal residues was, therefore, given consideration. Heating in boiling water for two minutes before reaction with FDNB or carrying out the reaction in *M* LiCl in 50:50 ethanol water mixtures or in 2 *M* sodium perchlorate (19) had no significant effect on the number of N-terminal residues found either in the original collagen or in the collagen buffered to pH 3.0 and stored at 75 percent R.H. (i.e. the most drastic conditions under which no obvious denaturation occurred during storage). Denaturation in itself does not, therefore, appear to be a factor influencing detection of N-terminal residues released. Denaturation in boiling water before buffering caused some increase in the N-terminal residues released during subsequent storage (0.41 to 0.72 millimole per 100 g in collagen stored at pH 3.0 and 75 percent R.H.). It seems probable that denaturation is a consequence of fairly extensive hydrolytic breakdown, but once having occurred it accelerates further hydrolytic action (cf. Hey and Stainsby (20) on the effect of alcohol on the degradation of collagen by strong alkalies).

Oxidative Breakdown.—The relatively high solubility of some of the collagen samples in relation to the number of N-terminal residues released suggested that some other type of breakdown had occurred. In earlier work on the solubility and amino acid composition of collagen stored at 100 percent R.H. and 40° or 60°C., it was suggested that some type of breakdown involving deamination occurred, possibly by an oxidative mechanism similar to that occurring on irradiation with γ-rays (4). Keto acids are postulated as products in such a reaction (5–7) but attempts to detect and determine these in the degraded collagens were not very successful largely owing to the large blank due to the collagen itself. Increases arising from storage, appear to be small, say a maximum of one millimole per 100 g.

Ferric ions are generally considered to catalyze oxidative breakdown. Soaking in ferric chloride solution to give final iron contents in the protein of 0.1 and 1 percent causes no increase in the number of keto groups detected after subsequent storage for eight weeks at 40°C.

TABLE V

KETO ACIDS IN DETERIORATED COLLAGENS

m. moles per 100 g as Pyruvic Acid

Collagen — before storage	5.2
Collagen — stored at 100% r. h. 40°C.	5.9
Collagen — stored at 100% r. h. 60°C.	5.3
Collagen — stored at 100% r. h. 40° + 0.1% $FeCl_3$	4.9
+ 1.0% $FeCl_3$	4.9
Collagen treated with acid H_2O_2	14.1

Another sample was treated by a modification of the peroxide test for deterioration, involving moistening with *N* sulfuric acid and treating three times with ten volume hydrogen peroxide, the sample being dried between each of these. The collagen appeared very degraded and on hydrolysis with 2 *N* hydrochloric acid for 16 hours at 105°C. reaction with DNPH indicated the presence of 14 millimoles per 100 g. Thus, under these more drastic conditions appreciable amounts of keto groups are formed and can be readily detected.

In view of these largely negative findings it was not considered worthwhile undertaking a more detailed study of the method involving separation of the individual dinitrophenyl hydrazones on the lines followed by Garrison et al. (7).

Chrome and Glutaraldehyde Tanned Collagen

Samples of collagen tanned with glutaraldehyde or chrome were stored under similar conditions and examined by the same procedures. The FDNB method cannot be applied to vegetable tanned leather (4).

Collagen tanned with glutaraldehyde and buffered with citrate showed appreciably less breakdown than collagen alone. (Results are included with those for collagen in Figure 1 and Table III.) There was only a slight deterioration in appearance, the number of N-terminal residues was reduced by a factor of four and the solubility was only about a tenth of that of collagen treated under similar conditions.

With chrome leather, buffering with citrate was impossible owing to the detanning effect of organic acid anions and the pH had to be adjusted with sulfuric acid. Even with this acid, however, some detanning will occur at the lower pH values due to displacement of chromium from the carboxyl groups by hydrogen ions. It was considered that the presence of salt might influence deterioration by reducing swelling and effective acidity. Two series were, therefore run, one with ten percent sodium chloride added to the acid used for adjusting the pH and one without.

A fifth series using the material of high chrome content studied in Part V was also included, together with a few further tests on collagen and glutaraldehyde tanned leather using sulfuric acid. Comparison with the citrate buffered series was considered unjustifiable because of the possible influence of the citrate ion on the breakdown.

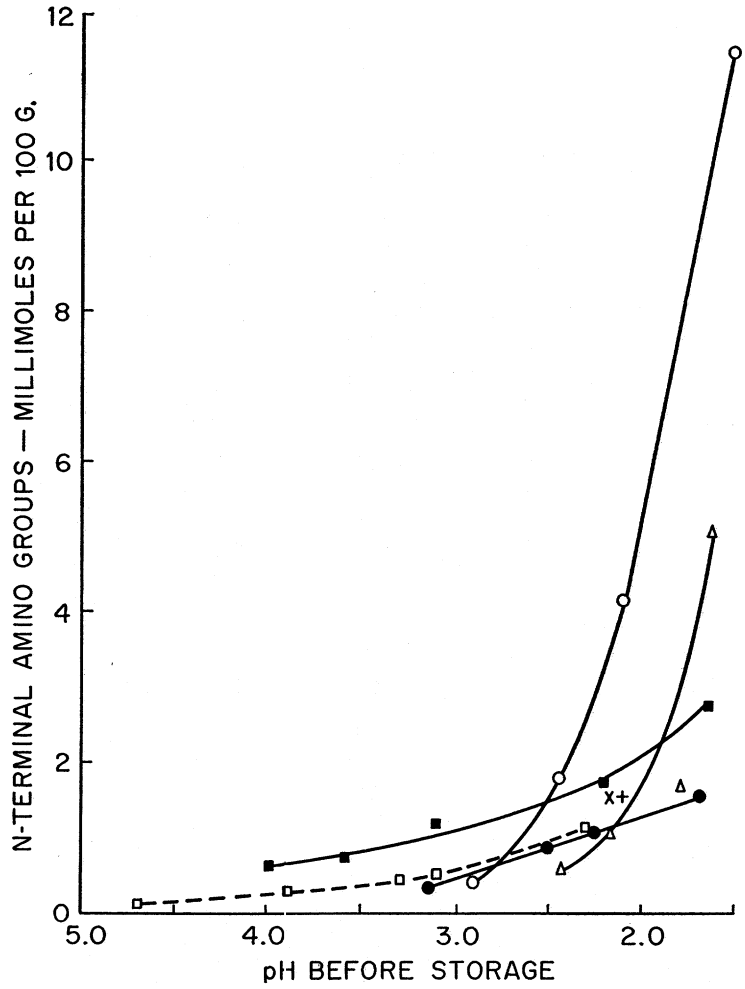

FIGURE 2.—N-terminal residues in chrome and glutaraldehyde tanned collagen stored at 100% r. h. and 40°C. for 8 weeks — unbuffered systems.
Chrome tanned — 0.57% Cr_2O_3 — ○——○ no salt; ●——● 5% NaCl
Chrome tanned — 4.0% Cr_2O_3 — △——△ no salt; △ (single point, off curve) 5% NaCl
Chrome tanned — 4.6% Cr_2O_3 — ■——■ no salt
Glutaraldehyde tanned collagen (two levels) □——□
Untanned collagen (single points) — + no salt; x 5% NaCl
Legends — Abscissa — pH before storage
Ordinate N-Terminal Amino Groups — Millimoles per 100 g

Results obtained are given in Table VI and Figure 2. They are less satisfactory and less amenable to interpretation than those obtained with the buffered systems since release of ammonia from amide groups and uptake of acid by the released amino groups led to appreciable increases in pH. As a result they were only exposed to low pH values for a relatively short period and the breakdown tends to be self limiting.

TABLE VI

SOLUBILITY AND N-TERMINAL RESIDUES IN CHROME AND GLUTARALDEHYDE TANNED COLLAGEN TREATED WITH SULPHURIC ACID AND STORED AT 100% R. H. AND 40°C.

	Normality of Acid	pH Range		Solubility, %		N-Terminal Residues m. mole per 100 g	
		No Salt	Salt	No Salt	Salt	No Salt	Salt
Collagen							
B	0.25	2.1–4.2	2.2–4.9	23	23	1.48	1.47
Glutaraldehyde — low level							
B	0.2	2.3–3.2	—	5	—	0.72	—
D	0.08	3.1–3.8	—	1	—	0.45	—
E	0.03	3.9–4.5	—	trace	—	0.30	—
Glutaraldehyde — high level							
B	0.2	2.3–3.1	—	1	—	1.02	—
D	0.08	3.3–3.8	—	1	—	0.47	—
F	0.03	4.7–4.9	—	4	—	0.19	—
F	0	7.7–7.3	—	2	—	0.23	—
Chrome — 0.57% Cr_2O_3							
A	0.5	1.5–4.2	1.7–4.5	54	5	11.5	1.61
B	0.15	2.1–4.0	2.2–4.0	7	1	2.14	1.07
C	0.08	2.5–2.8	2.5–4.1	1	1	0.77	0.89
D	0.04	3.0–3.5	3.1–4.3	1	1	0.41	0.30
Chrome — 4.0% Cr_2O_3							
A	0.5	1.6–3.9	1.8–4.2	11	2	5.03	1.14
B	0.15	2.1–3.3	—	2	—	1.03	—
C	0.08	2.5–3.0	—	trace	—	0.65	—
Chrome — 4.6% Cr_2O_3 (as used in Part IV)							
A	1.5	1.6–4.1		15	—	2.69	—
B	0.25	2.2–3.9		2	—	1.72	—
D	0.05	3.2–3.9		2	—	1.20	—
E	0.025	3.6–4.0		0.6	—	0.75	—
F	0	4.0–4.0		0.6	—	0.61	—

Letters indicate approximate initial pH i.e.
A = 1.5–1.6 D = 3.0–3.5
B = 2.1–2.4 E = 3.5–4.0
C = 2.5–3.0 F = >4
These are given to facilitate comparisons.

The glutaraldehyde tanned leathers in this series again showed little damage and hydrolytic breakdown was relatively slight, largely due to the rapid rise in pH. As far as comparison is possible the results suggest that as before tannage tends to reduce the number of N-terminal residues released.

The chrome tanned samples of collagen also showed relatively little damage. Although all those of low chrome content (0.57 percent Cr_2O_3) showed signs of darkening, only that stored at the lowest pH value without salt was extensively damaged. The samples stored with salt at the lowest pH and that stored without salt at the next lowest pH showed slight signs of damage. Of the samples tanned to the higher chrome content (4 percent Cr_2O_3) only that adjusted to the lowest pH value with no salt was affected.

The number of N-terminal residues increased with decrease in the initial pH of storage in all the series. Increases, however, became marked only when the initial pH value was about 2.0 and were less with the higher chrome content and in the presence of salt. The effect of salt may be related to reduced swelling during the acid treatment and hence to less unbound acid being held by the sample with consequent more rapid rise in pH during storage. Solubility followed the same general pattern, but the effect of the chromium in stabilizing the protein even when quite extensive hydrolytic breakdown has occurred was clearly apparent.

On the basis of the two results obtained with collagen adjusted to pH 3.3 with sulfuric acid it would seem that tannage has not greatly reduced susceptibility to hydrolysis. However, the initial pH of these two samples lies above that which appears to be critical for breakdown and it is also likely that the pH tends to rise more rapidly and to a higher level with collagen than it does with the tanned samples, thus limiting breakdown at an earlier stage. The fact that fewer N-terminal residues were released with the sample of high chromic oxide content than with the low indicates that tannage has in fact limited hydrolytic breakdown as well as greatly limiting subsequent solubility.

Tannage had no obvious influence on the proportions of the different N-terminal residues liberated, the general pattern being the same as with the buffered collagen.

DISCUSSION

It is clearly evident from the results that the breakdown of both collagen and tanned collagen occurs under warm moist conditions, the extent of the breakdown being dependent on both pH and humidity. Such breakdown becomes serious only when the humidity is high and the pH remains below about 3.0 for fairly long periods. It should be emphasized that all these tests were carried out at 40°C. and at lower temperatures the degree of damage, while almost certainly following the same general pattern, would be much less.

The possibility of some breakdown due to bacterial action cannot be entirely excluded in spite of the precautions taken. Such action would be most apparent at the higher pH values. The number of terminal residues released under these conditions is small, so it seems that bacterial breakdown, even if it does occur to a small extent, cannot have any serious influence on the conclusions.

Both hydrolytic scission of the chains and denaturation with loss of helical structure play a part in the degradation resulting from warm moist storage.

Broadly speaking, with untanned collagen solubility follows the same pattern as hydrolytic scission of the chains, increasing markedly at much the same pH as the sharp increase in N-terminal residues and first becoming obvious when these reach a value of about one millimole per 100 g. The general evidence suggests that hydrolytic breakdown is the first step; when this reaches a certain stage denaturation begins to occur and this, in its turn, is followed by some acceleration of the hydrolytic action possibly because of increased water uptake and accessibility of the peptide bonds.

The results obtained with the glutaraldehyde tanned hide powder buffered with citrate show that tanning, by helping to retain the structure, retards hydrolytic breakdown and what is perhaps more important, greatly reduces the solubility to be expected from the degree of hydrolytic breakdown which has occurred — compare for example collagen buffered to pH 3.5 stored at 100 percent R.H. containing 1.76 millimoles N-terminal residues per 100 g and 66 percent soluble, with glutaraldehyde tanned collagen buffered to pH 2.7 containing 2.67 millimoles N-terminal residues but only ten percent soluble.

The part played by chrome tanning is less clear since the rise in pH in the unbuffered series complicates interpretation. With initial pH values below about 2.2, hydrolytic breakdown is limited by the presence of chrome, the leather of higher chromic oxide content showing much less hydrolytic breakdown than that of the lower. With higher initial pH values there is no direct evidence that tanning with chromium reduces hydrolytic breakdown, but it is probable that this lack of effect is associated with the relative rate at which the pH rises during storage; the chromium complexes by exerting a buffering action reduce the rise in pH and prolong the time of exposure at the lower values, thus increasing breakdown relative to collagen stored at the same initial pH.

Solubility is clearly decreased by the presence of tan, the cross-links introduced helping to maintain the molecular structure in spite of hydrolytic scission. Here again the full effect of tanning is probably masked by the relative times of exposure at the low pH values.

To sum up, therefore, storage at low pH values and high humidities leads to hydrolytic breakdown of the collagen; as this becomes extensive it is followed by loss of structure, which in its turn accelerates further scission of the peptide

bonds. Cross-linking of the protein by a tanning agent retards hydrolytic breakdown during storage and helps to maintain structure even when such breakdown is quite extensive (see also ref. [3]).

The mechanism of the breakdown of bookbinding and upholstery leathers in acid atmospheres now becomes clearer. The degree of breakdown which occurs has often seemed out of proportion to the pH of water extract of the leather; now it can be seen that provided acid is continually supplied to the leather to replace that neutralized by the terminal residues released, hydrolytic breakdown can be quite appreciable even at pH 3.0. The humidity and temperature at which these leathers are normally kept are relatively low but the period of storage is very long. At 75 percent R.H., 40°C. and pH 3.0, about 0.5 millimole terminal residues per 100 g are released in eight weeks (see Figure 1), and it would be not unreasonable to assume that 1.5 millimoles would be liberated in a year at 30°C.

Vegetable tan, which relies on multiple weak bonds for its attachment to collagen, is not very efficient in maintaining the molecular structure of collagen and degradation of the leather follows the hydrolytic breakdown. Chromium tannage which introduces bonds relatively stable to moist heat is, however, able to prevent obvious deterioration even when hydrolytic breakdown is quite extensive.

Finally these results are relevant to pickling pelts for storage; at the acidities usually used for storage (pH of water extract 2.0–2.4) the dangers of hydrolytic scission of the peptide bonds is obvious. The presence of high concentrations of salt probably retards this to some extent, both by decreasing effective acidity and retarding denaturation, but the desirability is obvious of avoiding high temperatures and of keeping acidity as low as is compatible with control of mold growth and bacterial action.

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